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# Standard Practice for Measurement of the Biological Activity of Ricin[1]

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## INTRODUCTION

Ricin is a member of the protein toxins that cause their physiological effect by inactivation of ribosomes. Ricin is a member of the class 2 ribosome inactivating proteins (1).[2] Other members of this class of toxins include the proteins abrin and Shiga toxin.

Ricin consists of two chains, the A-chain that is responsible for the N-glycosidase enzymatic activity and the B-chain that is needed for cell binding and intra-cellular processing. Ricin is a heterogeneous protein with molecular weights ranging from approximately 62 to 64 kilodaltons (kDa) (2). Both chains are glycosylated and of similar size (approximately 32 kDa). There are several genes encoding putative ricin and ricin-like proteins in the genome of R. communis (3) resulting in differences in the amino acid sequence of the subunits. The differences in amino acid sequence and glycosylation both contribute to the heterogeneity of ricin.

## 1. Scope

1.1 This guide is intended for the manufacturers and users of ricin reference material. Ricin reference materials are well-characterized materials that can be used to test detection devices and calibrate laboratory measurements. It is anticipated that ricin reference materials will be characterized by biochemical methods in addition to the measurement of biological activity.

1.2 This practice details the measurement of ricin biological activity using a cell-free translation (CFT) assay (4).

1.3 The CFT assay has been developed for use in any biotechnology laboratory where determination or confirmation of ricin biological activity is required.

1.4 The CFT assay has been validated by the U.S. Army Medical Research Institute of Infectious Diseases (USAMRIID) VP-016 Validation of Cell-Free Translation Assay for the Detection of Ricin Toxin Biological Activities in compliance (5) with Good Laboratory Practices (GLP) Regulations of the Food and Drug Administration (21 CFR Part 58). Strict adherence to the protocol is necessary for validity of the test results.

1.5 Appendix X1 and Appendix X2 also provide guidance for the measurement of the biological activity of ricin using cell-based assays and the use of synthetic enzyme substrates.

1.6 Ricin is a category 2 select agent and acquisition of the ricin standard must adhere to the Center for Disease Control (CDC) regulations. Ricin is listed on the select agent list (42 CFR Part 72).[3] The possession, transfer, and use of ricin are restricted under the Public Health Security Preparedness Act (CRS Report RL31263 Public Health Security and Bioterrorism Preparedness and Response Act (P.L. 107-188): Provision and Changes to Preexisting law). Access to stores of ricin is limited (USA Patriot Act, P.L. 107-56). Ricin is also a prohibited substance under the Biological Weapons Convention and the Chemical Weapons Convention (CRS Report RL31559 Proliferation Control Regimes: Background and Status).

1.7 The values stated in SI units are to be regarded as standard. No other units of measurement are included in this standard.

1.8 *This standard does not purport to address all of the safety concerns, if any, associated with its use. It is the responsibility of the user of this standard to establish appropriate safety and health practices and determine the applicability of regulatory limitations prior to use.* Ricin is an extremely dangerous toxin. See Section 9 for specific hazards information.

[1] This practice is under the jurisdiction of ASTM Committee E54 on Homeland Security Applications and is the direct responsibility of Subcommittee E54.01 on CBRNE Sensors and Detectors.

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[2] The boldface numbers in parenthesis refer to the list of references at the end of this standard.

[3] Available at http://www.bt.cdc.gov/Agent/agentlist.asp.

## 2. Referenced Documents

2.1 *ASTM Standards:*[4]

F2149 Test Method for Automated Analyses of Cells—the Electrical Sensing Zone Method of Enumerating and Sizing Single Cell Suspensions

2.2 *Code of Federal Regulations:*

21 CFR Part 58 Good laboratory practice for nonclinical laboratory studies[5]

42 CFR Part 72 Interstate shipment of etiologic agents[6]

2.3 *ANSI/ATCC Standard:*[7]

ASN-0001-2009 Standardization of In-Vitro Assays to Determine Anthrax Toxin Activities

## 3. Terminology

3.1 *Abbreviations:*

3.1.1 *CFT*—cell free translation.

3.1.2 *CPS*—counts per second, units of luminescence instrument.

3.1.3 *IC50*—concentration of ricin that produces inhibition of 50 % of the activity in an assay.

3.1.4 *kDa*—molecular mass in kilo Dalton units.

3.1.5 *PBS*—phosphate buffered saline.

## 4. Summary of Practice

4.1 The CFT assay for measuring biologically active ricin is based on its inhibitory effects on protein synthesis (6, 7). When added to a rabbit reticulocyte translation mixture containing luciferase mRNA, ricin inhibits translation of the mRNA into the enzyme luciferase. Luciferase is then detected using a buffer containing the luciferin substrate. The test is a bioluminescence assay that measures the amount of luminescence proportional to the amount of luciferase produced from protein translation (RNA → protein). When active ricin is present, the amount of luminescence decreases corresponding to a decrease in the production of the luciferase enzyme. The amount of protein (luciferase) produced is directly proportional to the amount of luminescence generated. The decrease in luminescence is directly proportional to the amount of active ricin in the sample. Confirmation that translation inhibition is caused by the presence of active ricin is determined by mixing an aliquot of the ricin samples with anti-ricin antibody before adding to the translation mixture. The neutralized ricin does not inhibit luciferase translation, and therefore, luminescence does not decrease.

4.2 Cell-based assays use mammalian cells maintained in culture to measure the effect of ricin on cell death or damage (cytotoxicity). Ricin is added to the cells and after an incubation period, the effect on cell cytotoxicity is measured. The ricin-treated cells are compared to control cells (without added ricin) maintained under the same conditions. Guidance is given in Appendix X1.

4.3 The N-glycosidase enzymatic activity of the A-chain of ricin can be measured using synthetic oligonucleotides. The enzyme activity is measured either by the released adenine or the effect on the depurinated substrate using a number of methods. Guidance is given in Appendix X2.

## 5. Significance and Use

5.1 The CFT assay provides a sensitive and reliable method to detect ricin biological activity and results can be generated within 3 h. The assay measures the amount of ricin biological activity when compared to a known ricin standard and provides a quantitative measurement for active ricin.

5.2 The lower limit of quantitation and the upper limit of quantitation for ricin using the CFT assay was measured at 10 ng/mL and 170 ng/mL, respectively (5).

5.3 This practice is focused on the measurement of reference materials and not environmental samples. Additional control runs may be needed for measurements of environmental samples to ensure that the presence of additional materials in the samples (also referred to as the matrix) will interfere with the measurements.

5.4 The CFT assay may be used to determine the presence of active ricin in forensic or bioterrorist samples if the appropriate controls are utilized to ensure valid results (5).

5.5 The methods described in this document measure the biological activity of ricin and do not detect the presence of inactivated ricin in a given sample.

5.6 Ricin reference materials have a number of applications, such as testing detection devices, laboratory instruments, environmental sampling methods, disinfection studies, and basic research.

## 6. Apparatus

6.1 *List of Equipment*—The make and model are provided as examples, however equivalent apparatus may also be used.

6.1.1 *Mixer,* vortex mixing motion

6.1.2 *Display timers.*

6.1.3 *Incubator,* capable of maintaining temperature of (37 ± 1°C).

6.1.4 *96 Well Microplate Luminometer and Luminescence Test Plate.*[8]

[4] For referenced ASTM standards, visit the ASTM website, www.astm.org, or contact ASTM Customer Service at service@astm.org. For *Annual Book of ASTM Standards* volume information, refer to the standard's Document Summary page on the ASTM website.

[5] Available from Food and Drug Administration (FDA), 5600 Fishers Ln., Rockville, MD 20857, http://www.fda.gov.

[6] Available from U.S. Government Printing Office Superintendent of Documents, 732 N. Capitol St., NW, Mail Stop: SDE, Washington, DC 20401, http://www.access.gpo.gov.

[7] Available from American National Standards Institute (ANSI), 25 W. 43rd St., 4th Floor, New York, NY 10036, http://www.ansi.org.

[8] The sole source of supply of the apparatus (MicroLumi XS) known to the committee at this time is Harta Instruments, Inc., 8 Russell Ave Unit 106, Gaithersburg, MD 20877, www.hartainstruments.com. If you are aware of alternative suppliers, please provide this information to ASTM International Headquarters. Your comments will receive careful consideration at a meeting of the responsible technical committee,[1] which you may attend.

6.1.5 *Microplate Data Analysis Software,* KC4, with PowerReports,™ v3.0.[9]

6.1.6 *Plateshake.* [10]

6.1.7 *Laboratory Refrigerators (4°C), Freezer (-20°C), and Ultralow Freezer (-70°C or lower).*

6.1.8 *Water Bath,* 37 ± 1°C.

6.1.9 *Fixed Volume Pipettes, 1000 µL (200 to 1000 µL), 200 µL (20 to 200 µL), 20 µL (5 to 20 µL), 10 µL (1 to 10 µL), and 2 µL (0.5 to 2 µL), or adjustable pipettes of this range*—Pipettes should be regularly calibrated to ensure accurate dispensing of the set volumes.

6.1.10 *Multi-Channel Pipettes including 12-Channel (20 to 200 µL), 8-Channel Pipettor (2 to 20 µL), and 8-Channel pipettor (5 to 50 µL)*—Pipettes should be regularly calibrated to ensure accurate dispensing of the set volumes.

## 7. Reagents

7.1 *Reagents for the CFT Assay*—The validation of the assay was performed with reagents purchased from the specific vendors. The reproducibility and precision of the assay is dependent upon the quality of the reagents. The specific reagents have been tested to work in the validated assay. Substitution of reagents will require testing to ensure the same performance.

7.2 *Rabbit Reticulocyte Lysate,* nuclease treated.[11] The rabbit reticulocyte lysate is prepared from New Zealand white rabbits using a standard protocol under quality-controlled conditions (8). After the reticulocytes are lysed, the lysate is treated with micrococcal nuclease in order to destroy endogenous mRNA. The lysate is further optimized for mRNA translation by addition of an energy generating system, a mixture of tRNAs, hemin (to prevent inhibition of initiation), potassium acetate, and magnesium acetate. The rabbit reticulocyte lysate contains no endogenous mRNA and therefore translates only the mRNA added to the lysate (9). The ability to translate only one protein that can be detected permits a more accurate analysis of the biological effect of ricin's enzymatic reaction.

7.3 *Amino Acid Mixture, Complete*—The amino acid mixture, complete, has been prepared for use in the rabbit reticulocyte lysate systems and is an aqueous solution containing 1 m*M* each of the 20 essential amino acids. The mixture is sterile and RNA-free.

7.4 *Luciferase Control RNA*[11]—Luciferase control RNA used in these studies is commercially made using SP6 RNA polymerase transcription of a plasmid bearing the coding region for the luciferase gene with an additional 30 adenine residues that creates an uncapped, polyadenylated mRNA. The product of this luciferase control RNA is a monomeric protein (61 kDa) that does not require post-translational processing or modification for enzymatic activity. Only full-length luciferase is active. In most laboratories, contamination with extraneous luciferase or luciferase mRNA does not occur because luciferase is not found in laboratory environments.

7.5 *Luciferase Reporter Buffer*[11]—The luciferin reaction system is purchased as two components, lyophilized assay reagent and assay buffer. When mixed, the luciferase assay buffer provides high quantum efficiency and no background luminescence in the reticulocyte system or in the assay chemistry. Light is produced by converting the chemical energy of luciferin oxidation through an electron transition, forming the product molecule of oxyluciferin. The luciferase reaction buffer contains coenzyme A that improves kinetics and allows for greater enzymatic turnover resulting in increased light intensity. Unlike many chemiluminescent reactions, this reaction remains constant for nearly two minutes, thereby permitting accurate measurements using a microtiter format (4). As noted, the amount of luminescence emitted with this reaction buffer is proportional to the amount of luciferase present and therefore provided a comparative measurement of the luciferase amount in samples treated with toxins.

7.6 *RNase Inhibitor*[11], a 50 kDa protein that noncovalently binds to RNases in a 1:1 ratio, is a broad-spectrum RNase inhibitor. The product is purified using a combination of ion exchange and affinity chromatography.

7.7 *Sterile Nuclease-Free De-ionized Water*[11], sterile and RNase free.

7.8 *Ricin Standard and Antibody*—Reagents should be comparable to the following products:

7.8.1 *Ricin Toxin (Ricin), Ricinus Communis Agglutinin II, 5 mg/mL*[12]—This product, purified from castor beans, is the approximately 60 kDa molecular weight protein that is highly toxic with little agglutinin activity. The toxin is purchased as a liquid (5 mg/mL) and contains 0.08 % sodium azide. At this concentration, the sodium azide does not affect the assay.

7.8.2 *Antibody to Ricinus Communis Agglutinin I and II, Affinity Purified from Goat, 2 mg/mL*[12], This product is a polyclonal anti-ricin IgG and provides excellent neutralization of ricin toxin.

[9] The sole source of supply of the apparatus known to the committee at this time is BioTek, P.O. Box 998, Highland Park, Winooski, VT 05404, http://www.biotek.com/. If you are aware of alternative suppliers, please provide this information to ASTM International Headquarters. Your comments will receive careful consideration at a meeting of the responsible technical committee,[1] which you may attend.

[10] The sole source of supply of the apparatus (DELFIA (Dissociation-Enhanced Lanthanide Fluorescent Immunoassay), product # 1296-003) known to the committee at this time is PerkinElmer, 940 Winter St., Waltham MA 02451, http://www.perkinelmer.com/. If you are aware of alternative suppliers, please provide this information to ASTM International Headquarters. Your comments will receive careful consideration at a meeting of the responsible technical committee,[1] which you may attend.

[11] The sole source of supply of the reagent known to the committee at this time is Promega Corporation, 2800 Woods Hollow Rd., Madison, WI 53711, www.promega.com. If you are aware of alternative suppliers, please provide this information to ASTM International Headquarters. Your comments will receive careful consideration at a meeting of the responsible technical committee,[1] which you may attend.

[12] The sole source of supply of the reagent known to the committee at this time is Vector Laboratories, 30 Ingold Rd., Burlington, CA 94010, www.vectorlabs.com. If you are aware of alternative suppliers, please provide this information to ASTM International Headquarters. Your comments will receive careful consideration at a meeting of the responsible technical committee,[1] which you may attend.

7.9 *Phosphate Buffered Saline (PBS)*[13], with a pH of 7.2. Sterile PBS solutions were tested in the CFT assay and this PBS did not interfere with the assay.

7.10 *Polyoxethylenesorbitan Monolaurate*[13], molecular biology grade, ensured to be endonuclease, exonuclease, and RNAse free.

7.11 *Disinfectant*—Bleach, 5 % sodium hypochlorite. Ensure that the expiration date on the bleach has not passed and that the bleach is still active.

## 8. Materials

8.1 *96 V Shaped Bottom Plates,* sterile, tissue culture grade.

8.2 *96 Well Flat Bottom Plate,* black with clear bottom.

8.3 *Sterile Pipette Tip.*

8.4 *15 mL and 50-mL Conical Tubes,* sterile.

8.5 *1.5 mL Microtubes,* sterile.

8.6 *100 mL Disposable Reagent Reservoir,* sterile.

8.7 *Low-Density Polyethylene Bag,* zip-lip, 5 by 8 in.

8.8 *250 mL Glass Bottle,* sterile, for preparing buffer.

8.9 *Ice Bucket (Ice Pan).*

8.10 *Waste Container.*

8.11 *Paper Towels,* disposable.

8.12 *Nitrile Gloves,* nonsterile.

## 9. Hazards

9.1 Ricin is extremely toxic in very small quantities (10, 11). The user of this practice is responsible for ensuring that the procedures are carried in full compliance with the institute's safety/biosurety regulations.

9.2 Ricin should be managed as a hazardous chemical and included in the laboratory specific chemical hygiene plan. Ricin in powder form is more hazardous, therefore work with ricin powder should be avoided if possible. The institute's biosurety/biosafety office should be contacted prior to any work with powder samples containing (or thought to contain) ricin.

9.3 The use of ricin requires biosafety level 2 (BSL2) or higher and specialized procedures including training for working with toxins of biological origin (12).

9.4 Any materials containing ricin waste should be inactivated by autoclaving or treating with active solutions of bleach (10 % vol/vol) for 30 min before disposal.

## 10. Calibration and Standardization

10.1 Calibration of instruments should be performed in accordance with the institute's standard operating procedures or manufacturer's instructions.

10.2 The assay is standardized using the reagents listed. If different reagents are used, standardization of the materials is required.

10.3 The performance and concentration of the anti-ricin antibody used should be determined.

10.4 The purity and concentration of the ricin standard used should be verified by analytical measurements.

10.4.1 Denaturing gel electrophoresis using sodium dodecyl sulfate (SDS) and conditions that reduce the disulfide bond between the A and B subunits, results in two bands of approximately 32 kDa (13).

10.4.2 Techniques such as size exclusion chromatography can also be used to determine the heterogeneity of the ricin sample, pure samples should give a single peak with an approximate molecular weight of 60 kDa (14).

10.4.3 Other methods can be used for characterizing ricin preparations such as ion-exchange chromatography and isoelectric focusing (15).

10.5 The ricin protein concentration of pure ricin samples should be determined for reference samples. The molar absorption coefficient of ricin in phosphate-buffered saline (PBS) at 279 nm was measured as (93 900 ± 3300) L $mol^{-1}$ $cm^{-1}$, amino acid analysis is used to determine the protein concentration (16).

## 11. Procedure

11.1 *Preparation of CFT Luciferase Reaction Buffer:*

11.1.1 Thaw the luciferase assay buffer using a 37°C water bath.

11.1.2 Add the thawed assay buffer to the luciferase assay substrate powder that is contained in a brown glass bottle. Dispense 10 mL to each tube of the reaction buffer into conical tubes. Each tube (10 mL) is enough for 2 plates.

11.1.3 Assign a lot number and label the tube with the name (luciferin assay buffer), the date made, and the expiration date (1 month if stored at -20 ± 10°C, or 1 year if stored at –70 ± 10°C after the preparation date). Wrap each tube with aluminum foil and store the buffer at a -20°C or -70°C.

11.2 *Preparation of the Assay Buffer (PBS Containing 0.02 % Tween 20):*

11.2.1 Pipette 200 mL of PBS into a sterile glass bottle and add 40 µl Tween 20. Mix gently to insure that Tween 20 is dispersed. Larger volumes can be prepared as long as sterility of the container is maintained.

11.2.1.1 Prepare assay buffer fresh each day.

11.3 *Preparation of Ricin Standard Working Solution (0.5 µg/mL):*

11.3.1 Add 5 µL of ricin (5 mg/mL) to 2495 µL of assay buffer to make ricin stock solution (10 µg/mL). Label the stock solution and store at 4°C. The stock solution is stable for 2 weeks when stored at 4°C (5), but caution should be exercised in storage of dilute concentrations of ricin to confirm the stability or if losses due to adsorption to storage containers have occurred. When in doubt prepare a fresh solution from the concentrated stock.

11.3.2 Then add 50 µL of the stock solution to 950 µL assay buffer to prepare a 0.5 µg/mL ricin working solution.

[13] The sole source of supply of the reagent known to the committee at this time is Sigma-Aldrich Corp., St. Louis, MO, www.sigmaaldrich.com. If you are aware of alternative suppliers, please provide this information to ASTM International Headquarters. Your comments will receive careful consideration at a meeting of the responsible technical committee,[1] which you may attend.

11.3.3 Place the ricin standard working solution (0.5 µg/mL) in ice pan.

11.4 *Preparation of Anti-Ricin Antibody Working (Neutralization) Solution (10 µg/mL):*

11.4.1 Add 10 µL goat anti-ricin antibody stock solution (2 mg/mL) to 1090 µL assay buffer for every set of 3 samples.

11.4.2 Keep anti-ricin antibody stock solution at 4°C.

11.5 *Equipment Check:*

11.5.1 Check to ensure that the incubator, vortex mixer, and shaker platform are working properly.

11.5.2 Turn on and test the luminometer following the manufacturer's users manual, using the luminescence filter and settings. Run the luminescence test plate to ensure that the instrument is functioning correctly.

11.6 *Prepare the Necessary Amount of Luciferase Reporter, Assay Buffer, and Reagents on Ice:*

11.6.1 Place the assay buffer, ricin working solution, anti-ricin antibody working solution, test samples, water, amino acid solution, and luciferase mRNA in the ice bath. Except for incubating ricin with antibody, all reagents shall be at a temperature between 0 to 4 in order to minimize translation before incubating the lysate.

11.7 *Serial Dilution of Ricin Standards and Unknown Test Samples:*

11.7.1 Seven concentrations of ricin have been characterized as providing a suitable sigmoid standard curve for this assay. The standards are prepared by serially diluting ricin working solution with assay buffer. The final concentrations of the standards are: S1 = 250 ng/mL, S2 = 125.0 ng/mL, S3 = 62.5 ng/mL, S4 = 31.3 ng/mL, S5 = 15.6 ng/mL, S6 = 7.8 ng/mL, and S7 = 3.9 ng/mL. The concentrations for the seven standards may need to be adjusted when using a different lot of ricin or ricin purchased from a different vendor. The ricin standards are prepared in separate tubes and then added to the plates, so that the same standards can be added to multiple plates if needed.

11.7.2 The starting dilution of test samples should be determined by the assay supervisor based on sample information or pre-test results before starting the assay. The amount used for serial dilution of unknown test samples varies, depending on quantity/amount of the sample.

11.7.3 Prepare two sets of serial dilutions for ricin standards (S), unknown test samples (T), and buffer control (BC) on a v-shaped 96-well microplate. One set will be used for CFT assay and the second set will be neutralized with ricin antibody and then analyzed by the assay to confirm ricin toxicity. The plate should be set up in accordance with Table 1.

11.7.4 Add 50 µL assay buffer to the wells for all of the BC samples, and the T samples in rows C, D, E, F, G, and H (but not row B, according to the configuration shown in Table 1).

11.7.5 Add 50 µL of the corresponding ricin standard (S1 to S9) to the wells of columns 1 and 9.

11.7.6 Add 100 µL of unknown sample starting dilution to each corresponding well of row B (columns 2, 3, 4, 10, 11, and 12).

11.7.7 Perform 2-fold serial dilution of unknown samples by transferring 50 µL/well from row B to row C, and so on continuing to row H. Remove and discard 50 µL from row H. After each transfer, mix dilutions by pipetting up and down 7 times. Clean pipette tips should be used after each serial dilution step

11.8 *Prepare Anti-Ricin Antibody Neutralization Reaction:*

11.8.1 Add 50 µL/well of the anti-ricin antibody neutralization working solution (10 µg/mL) to one set of standards, samples, and buffer control (columns 9-12). After each addition, mix solutions by pipetting up and down 5 times.

11.8.2 Add 50 µL/well of the assay buffer to the other set of standards, samples, and control (Columns 1-4). After each addition, mix solutions by pipetting up and down at least 5 times.

11.8.3 Cover and gently shake the plate on a shaker at room temperature (25 ± 3) for 20 ± 5 min.

11.9 *Transferring Solutions:*

11.9.1 Place a clean V-shaped bottom plate on ice in an ice bucket.

11.9.2 Remove the lysate plate from the shaker and place the plate on ice.

11.9.3 Transfer 5 µL/well from each set of standards, sample dilutions, and control to corresponding wells in a V-shaped bottom plate.

11.10 *Preparation of Translation Reagent:*

11.10.1 Place the reagent reservoir on ice in an ice bucket.

11.10.2 Prepare the translation reagent by adding the following reagents in the reagent reservoir.

11.10.3 All work should be done on ice (4 ± 2) in order to prevent the onset of translation.

11.10.4 Add reagents in the same order as listed in Table 2.

11.10.5 Mix reagents by pipetting the mixture gently up and down 5 times using a 1000 µL pipettor.

11.11 *Incubation of Translation/Ricin Mixture:*

**TABLE 1 Configuration for 96-well Microplate for Serial Dilution of Ricin Standards and Unknown Samples**

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | BC[A] | BC[A] | BC[A] | BC[A] | | | | | BC[A] | BC[A] | BC[A] | BC[A] |
| B | S1[B] | T1–1[C] | T2–1[C] | T3–1[C] | | | | | S1[B] | T1–1[C] | T2–1[C] | T3–1[C] |
| C | S2[B] | T1–2[C] | T2–2[C] | T3–2[C] | | | | | S2[B] | T1–2[C] | T2–2[C] | T3–2[C] |
| D | S3[B] | T1–3[C] | T2–3[C] | T3–3[C] | | | | | S3[B] | T1–3[C] | T2–3[C] | T3–3[C] |
| E | S4[B] | T1–4[C] | T2–4[C] | T3–4[C] | | | | | S4[B] | T1–4[C] | T2–4[C] | T3–4[C] |
| F | S5[B] | T1–5[C] | T2–5[C] | T3–5[C] | | | | | S5[B] | T1–5 | T2–5 | T3–5 |
| G | S6[B] | T1–6[C] | T2–6[C] | T3–6[C] | | | | | S6[B] | T1–6[C] | T2–6[C] | T3–6[C] |
| H | S7[B] | T1–7[C] | T2–7[C] | T3–7[C] | | | | | S7[B] | T1–7[C] | T2–7[C] | T3–7[C] |

[A] BC = buffer control.
[B] Ricin standard samples: S1 = 250 ng/mL, S2 = 125.0 ng/mL, S3 = 62.5 ng/mL, S4 = 31.3 ng/mL, S5 = 15.6 ng/mL, S6 = 7.8 ng/mL, and S7 = 3.9 ng/mL.
[C] Sample dilutions: T1, T2, and T3 = serial dilutions of unknown samples prepared in triplicate.

**TABLE 2 Translation Reagent Preparation**

| Reagent Name | Volume (per 3 unknown samples) |
|---|---|
| Nuclease-Free Water | 1000 µL |
| Rabbit Reticulocyte Lysate, Nuclease Treated | 1000 µL |
| RNasin RNase inhibitor | 5 µL |
| Amino Acid Mixture, Complete | 35 µL |
| Luciferase Control RNA | 30 µL |

11.11.1 Immediately add 25 µL of above mixed translation reagent solution into each well of the V-shaped plate containing 5 µL of the samples.

11.11.2 Gently tap the sides of the plate. Avoid creating air bubbles.

11.11.3 Place a damp paper towel around the plate and place the plate in a sealable plastic bag.

11.11.4 Incubate the plate at 37 ± 1°C for 90 ± 5 min.

11.11.5 After the 90 ± 5 min incubation, remove the plate from the plastic bag.

11.11.6 Immediately place the plate on ice in the ice pan. Leave on ice for 5 min.

11.11.7 *Transferring Reaction Mixture and Adding Luciferase Reaction Buffer:*

11.11.7.1 Transfer 5 µL per well of the reaction mixtures from the translation incubation plate to a new 96 well, clear bottom black plate.

11.11.7.2 All dilutions should be run in triplicate. Each set of samples is run on one black plate. One plate shall be used for the normal translation set and one plate for the anti-ricin antibody (neutralization) translation reaction set.

11.11.8 Set up black plates as shown in Table 3.

11.11.9 Without interruption, add 45 µL of luciferase reaction buffer to each well (in black plates) using the multichannel pipettes to the wells containing the 5 µL samples. Once the luciferase reaction buffer is added the plate shall be read by the luminometer within 2 min.

11.11.10 Gently tap the sides of the plate to mix.

11.12 *Reading the Plates:*

11.12.1 Immediately following the addition of the luciferase reporter buffer place the plate in the luminometer and read within 2 min.

11.12.2 Save raw data, counts per second (CPS) to a disk.

11.13 *Calculations:*

11.13.1 Calculate the mean CPS, standard deviation (SD), and %CV of the triplicate wells, including standards, controls, and samples, on each individual test black plate with the Bio-Tek microplate data analysis software with PowerReports™ (KC4, v3.0),[9] or a suitable statistical analysis software program.

11.13.2 The four parameters and $R^2$ of ricin standard curves and mean ricin concentration of sample dilutions are calculated with KC4 software, using the following four-parameter logistic (4PL) equation:

$$y = (a - d)/(1 + (x/c)b) + d \quad (1)$$

*where:*

$y$ = the expected response,
$x$ = concentration,
$a$ = response at zero concentration,
$d$ = response at infinite concentration,
$c$ = concentration resulting in a response halfway between $a$ and $d$ (the IC50), and
$b$ = slope parameter

11.13.3 Specific active ricin in the test samples is confirmed by comparing CPS of each sample dilution without and with the addition of anti-ricin IgG, which neutralized the ricin toxic activity.

11.13.4 Raw data (CPS) should be stored in a computer spreadsheet program.

11.13.5 Calculate percent recovery (% recovery), percent of relative error (% RE), and all mean concentrations, standard deviation (SD), and % CV for dilution-to-dilution, plate-to-plate, and day-to-day comparison, using the following equations:

$$\%\ CPS = CPS_{sample}/CPS_{buffer\ control}\ x\ 100 \quad (2)$$

$$\%\ recovery = observed/nominal\ x\ 100 \quad (3)$$

$$\%\ RE = (Observed/Nominal - 1)\ x\ 100 \quad (4)$$

$$\%\ CV = SD/mean\ x\ 100 \quad (5)$$

Nominal concentration refers to the initial concentration of the ricin used as the standard.

11.14 The P value is calculated by paired T-test for neutralization data and un-paired T-test for stability data, using a suitable statistical analysis software program (see Fig. 1).

## 12. Disposal of All Ricin Solutions

12.1 All liquid waste shall be decontaminated using a final concentration of 10 % bleach solution for a 30-min contact time.

**TABLE 3 Configuration for 96-well Black Micro Plates for the Luciferase Reaction**

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | BC | BC | BC | BC | BC | BC | BC | BC | BC | BC | BC | BC |
| B | S1 | S1 | S1 | T1–1 | T1–1 | T1–1 | T2–1 | T2–1 | T2–1 | T3–1 | T3–1 | T3–1 |
| C | S2 | S2 | S2 | T1–2 | T1–2 | T1–2 | T2–2 | T2–2 | T2–2 | T3–2 | T3–2 | T3–2 |
| D | S3 | S3 | S3 | T1–3 | T1–3 | T1–3 | T2–3 | T2–3 | T2–3 | T3–3 | T3–3 | T3–3 |
| E | S4 | S4 | S4 | T1–4 | T1–4 | T1–4 | T2–4 | T2–4 | T2–4 | T3–4 | T3–4 | T3–4 |
| F | S5 | S5 | S5 | T1–5 | T1–5 | T1–5 | T2–5 | T2–5 | T2–5 | T3–5 | T3–5 | T3–5 |
| G | S6 | S6 | S6 | T1–6 | T1–6 | T1–6 | T2–6 | T2–6 | T2–6 | T3–6 | T3–6 | T3–6 |
| H | S7 | S7 | S7 | T1–7 | T1–7 | T1–7 | T2–7 | T2–7 | T2–7 | T3–7 | T3–7 | T3–7 |

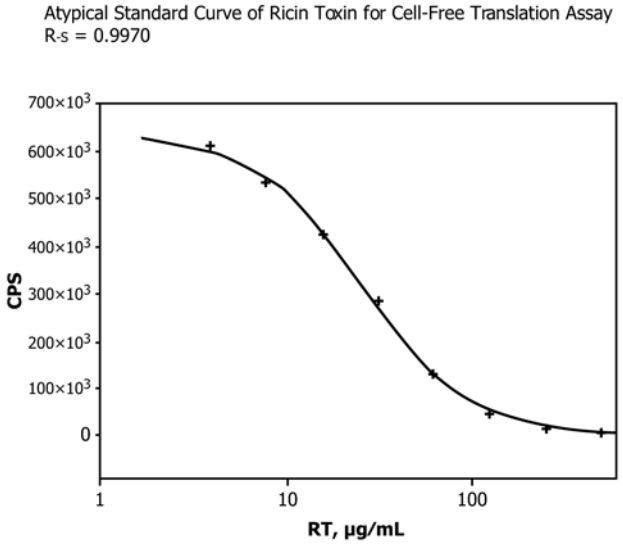


**FIG. 1 Atypical Standard Curve of Ricin Toxin for Cell-Free Translation Assay ($R_S$ = 0.9970)**

12.2 Dispose decontaminated materials in accordance with the institution's policies.

## 13. Report

13.1 *Acceptance Criteria for Plates*—Determine whether the test black plate is acceptable by recording Y or N in the spaces of Table 4.

13.2 *Acceptance Criteria/Concentration/Toxin Type for Unknown Test Samples:*

13.2.1 Record toxin concentration, %CV, and whether the toxin is confirmed to be ricin.

13.2.2 Record negative sample as " – " in the form shown in Table 5.

## 14. Keywords

14.1 biodefense; biological activity; cell culture; cell free translation (CFT) assay; enzymatic activity; forensic test; ricin; toxin

**TABLE 4 Acceptance Criteria for Plate Data Report**

| Criteria | Plate No. 1 | Plate No. 2 | Plate No. 3 |
|---|---|---|---|
| Standards $CV \leq 25$ % | | | |
| Control $CV \leq 20$ % | | | |
| Slope $B$ | | | |
| $R^2 > 0.99$ | | | |
| Plate Acceptable (if criteria listed above are met) | | | |

TABLE 5 Acceptance Criteria Report for Unknown Samples

| Test Sample ID | $CV \leq 20$ % | Toxin Concentration, µg/mL | Ricin Confirmed | Other Information |
|---|---|---|---|---|

## APPENDIXES

**(Nonmandatory Information)**

## X1. CELL BASED AND SYNTHETIC SUBSTRATE ASSAYS

### X1.1 Cells Used to Measure Ricin Activity

X1.1.1 Ricin is a galactose-specific toxin lectin that is taken into cells through the binding of the B chain to galactose-terminated molecules on the cell surface and also through mannose receptors expressed on the surface of macrophagic cells (17).

X1.1.2 A number of mammalian cell lines have been used to measure the cytotoxicity of ricin (18).

X1.1.3 Cells should be screened for contamination by microorganisms, such as mycoplasma, to ensure the purity and quality of the cell culture results

X1.1.4 Cells should be obtained from a reputable repository to ensure authenticity of the line.

X1.1.5 It is recommended that cells should be passaged no more than 30 times when used in measuring the activity of ricin, due to potential changes in genetic and phenotypic characteristics that are associated with cells with high passage numbers.

X1.1.6 Vero cells (African green monkey epithelial cells[14]) are used here as an example to demonstrate one method for measuring ricin activity in vitro.

### X1.2 Referenced Documents

X1.2.1 Polio Laboratory Manual, 4th edition, 2003, World Health Organization, Department of Immunization, Vaccines and Biologicals, CH-1211, Geneva 27, Switzerland.[15]

### X1.3 Terminology

X1.3.1 *IC50*—the concentration of ricin that produces an inhibition of cell viability (cytotoxicity) or decrease in protein synthesis by 50 %. This value is determined from a series of concentrations of ricin.

X1.3.2 *Definitions*—The following cell culture terms are from either the Health Protection Agency Culture Collections (NIH; UK)[16] or the National Institutes of Health (NIH) Chemical Genomics Center (NCGC).[17]

X1.3.2.1 *Cell Culture*—Establishment and maintenance of cultures derived from dispersed cells taken from original tissues, primary culture, or from a cell line or cell strain.

X1.3.2.2 *Continuous Cell Culture*—A culture that is apparently capable of an unlimited number of population doublings; often referred to as an *immortal cell culture*.

X1.3.2.3 *Immortalization*—The attainment by a finite cell culture, whether by perturbation or intrinsically, of the attributes of a continuous cell line. An immortalized cell is not necessarily one, which is neoplastically or malignantly transformed.

X1.3.2.4 *Passage*—The transfer or transplantation of cells, with or without dilution, from one culture vessel to another. It is understood that any time cells are transferred from one vessel to another, a certain portion of the cells may be lost and therefore, dilution of cells, whether deliberate or not, may occur. This term is synonymous with the term *subculture*.

X1.3.2.5 *Passage Number*—The number of times the cells in the culture have been subcultured or passed. In descriptions of this process, the ratio or dilution of the cells should be stated so that the relative cultural age gap can be ascertained.

X1.3.2.6 *Population Doubling Level*—The total number of population doublings of a cell line or strain since its initiation in vitro.

X1.3.2.7 *Primary Culture*—A culture started from cells, tissues, or organs taken directly from organisms. A primary culture may be regarded as such until it is successfully subcultured for the first time, when it becomes a *cell line*.

X1.3.2.8 *Tissue Culture*—Traditionally, the maintenance of fragments of tissue in vitro, but commonly is used as a generic term including tissue explant culture, organ culture, and dispersed cell cultures (for example, cell lines and cell strains).

### X1.4 Apparatus

X1.4.1 *Mixer,* vortex mixing motion.

X1.4.2 *Display Timers.*

X1.4.3 *Laboratory Refrigerators (4°C), Freezer (-20°C) and Ultralow Freezer (-70 to -80°C).*

X1.4.4 *Water Bath,* 37 ± 1 ºC.

X1.4.5 *Fixed Volume Pipettes*—1000 µL, 200 µL, 20 µL, 10 µL, and 2 µL or adjustable pipettes of similar range. Pipettes should be calibrated to ensure accurate dispensing of the set volumes.

X1.4.6 *Multi-Channel Pipettes,* including 12-channel (20 to 200 µL), 8-channel pipettor (2 to 20 µL) and 8-channel pipettor

[14] The sole source of supply of the cells (#CCL-81) known to the committee at this time is American Type Culture Collection (ATCC) , P.O. Box 1549, Manassas, VA 20108. If you are aware of alternative suppliers, please provide this information to ASTM International Headquarters. Your comments will receive careful consideration at a meeting of the responsible technical committee,[1] which you may attend.

[15] http://www.who.int/vaccines/en/poliolab/webhelp/

[16] http://www.hpacultures.org.uk/glossary/index.jsp

[17] http://www.ncgc.nih.gov/guidance/section7.html#cell-culture-glossary

(5 to 50 µL). Pipettes should be calibrated to ensure accurate dispensing of the set volumes.

X1.4.7 *Biological Safety Cabinet,* Class II, type A2, that provides a sterile environment for cell culture that will provide sterile air flow in the cabinet and inward flow of air to protect the user. The correct performance of the biological safety cabinet should be checked every time it is used. The user is responsible for ensuring the proper maintenance and certification of performance of the biological safety cabinet.

X1.4.8 *Tissue Culture Incubator,* capable of maintaining a temperature of 37 ± 1 ºC), a high relative humidity (95 %), and a $CO_2$ level of 5 % in air.

X1.4.9 *Cell Culture Flasks,* with sterile 25 and 75 $cm^2$ surface area for cell growth.

X1.4.10 *Cell Counting Slides,* such as a hemocytomer or a laboratory instrument to determine cell concentration.

### X1.5 Reagents

X1.5.1 *Cells used for Cytoxicity Measurement:*

X1.5.1.1 The choice of cells is important, since the sensitivity of cells to ricin is dependent on the binding of ricin to the surface of cells and intracellular processing.

X1.5.1.2 Permanent cells lines obtained from biological repositories provide a source of uniform characterized cells that can be used by a number of investigators.

X1.5.1.3 Cell lines have been engineered to express a protein that is easily measured and can be used to detect the effect of ricin on cellular protein synthesis. The enzyme luciferase expressed in Vero cells is used to measure ricin activity (18). The amount of luciferase produced in the cells is measured using an assay based on light production by the enzyme activity. The expression of Vero cells that have been engineered to express green fluorescent protein (GFP) is also used to measure the effect of ricin on protein synthesis (19). The decrease in cellular fluorescence in response to ricin was measured using fluorescence microscopy or with a flow cytometer.

X1.5.2 The purity, authenticity, and stability of the cells should be assessed prior to beginning the measurement of ricin biological activity for ricin.

X1.5.3 The purity and concentration of ricin material used as a standard should be confirmed as described in 7.

X1.5.4 The performance and purity of the cell culture media used should be confirmed by testing with cells of known properties.

X1.5.5 *Trypsin,* 0.05 % in 0.53 mM EDTA, sterile cell culture grade.

X1.5.6 *Fetal Bovine Serum,* cell culture grade (mycoplasma, virus, and endotoxin negative).

X1.5.7 *Eagle's Minimum Essential Media,* sterile, cell culture grade.

### X1.6 Procedure

X1.6.1 *Cell Culture of Vero Cells:*

X1.6.2 All cell work and media preparation should be performed in a sterile environment using a Class II Biological Safety Cabinet (BSL2 hood). Proper aseptic techniques should be used to avoid contamination events.

X1.6.3 The base medium for Vero cells is Eagle's Minimum Essential media. To make the complete growth medium, add fetal bovine serum to a final concentration of 10 % (vol/vol).

X1.6.4 Culture the cells in an incubator maintaining an atmosphere of 95 % air, 5 % $CO_2$, and 37°C.

X1.6.5 *Subculturing and Passaging:*

X1.6.5.1 This example is based on Vero cells cultured in T-75 flasks (75 $cm^2$).

X1.6.5.2 Remove and discard the culture medium. Briefly rinse the cell layer with 0.25 % (w/v) Trypsin- 0.53 mM EDTA solution to remove all traces of serum that contains trypsin inhibitor.

X1.6.5.3 Add 2.0 to 3.0 mL of Trypsin-EDTA solution to the flask and observe cells under an inverted microscope until the cell layer is dispersed (usually within 5 to 15 min). To avoid clumping do not dislodge the cells by hitting or shaking the flask while waiting for the cells to detach. Cells that are difficult to detach may be placed at 37°C to facilitate removal.

X1.6.5.4 Add 6.0 to 8.0 mL of complete growth medium and aspirate cells by gently pipetting and avoid creating bubbles.

X1.6.6 Enumeration of cells can be performed using a hemacytometer or a Coulter Counter (see F2149).

X1.6.7 Add appropriate aliquots of the cell suspension to new culture vessels. Incubate cultures at 37°C. A subcultivation ratio of 1:3 to 1:6 is recommended.

X1.6.8 Change the media 2 to 3 times per week as needed.

X1.6.9 *Crystal Violet Method to Measure Cytotoxicity:*

X1.6.9.1 The cytotoxic activity of ricin can be determined using the crystal violet method based on cell adhesion of viable cells (20). Cells that are intoxicated with ricin will detach from the plate and therefore will not be stained. Viable cells remain adherent and will be stained by the dye.

X1.6.9.2 The stain intensity is proportional to the number of viable, adherent cells in the well.

X1.6.9.3 For optimal results, do not use any reagents past the expiration date or suggested shelf life.

X1.6.9.4 On day 1, the cells (at passage less than 20) are seeded into a 96-well tissue culture plate (2x104 cells/well) and incubated overnight at 37°C and 5 % $CO_2$. Peripheral wells (columns 1 and 12 and rows A and H) should not be used due to the variability of cell growth that can be associated with the edges of the plate.

X1.6.9.5 Prepare a 0.5 mg/mL working solution of ricin in sterile PBS.

X1.6.9.6 In the first column of a clean, sterile 96 well microtiter plate, add 148.5 µL of complete growth medium and 1.5 µL of 0.5 mg/mL ricin toxin (resulting in a final concentration of 5 µg/mL of ricin). In all subsequent columns, add 135 µL of complete growth medium.

X1.6.9.7 Proceed with subsequent 10-fold dilutions by transferring 15 µL from the previous well into the next well

until well 11 is reached. Remember to discard the pipette tips between each dilution.

X1.6.9.8 Remove the media from plate with cells and transfer 100 µL media containing the diluted ricin (prepared in the previous step) to the corresponding wells.

X1.6.9.9 Include a set of wells that contain complete growth medium without toxin as a negative control.

X1.6.9.10 A set of control wells without cells should be read to obtain the background reading.

X1.6.9.11 Other controls should include antibody neutralization as a negative control prepared as described in the standard guide for the CFT assay for ricin described above.

X1.6.9.12 Cycloheximide can be used as positive control for cytoxicity (20).

X1.6.9.13 Culture the cells at 37°C, 5 % $CO_2$ for 48 h. Shorter incubation times can also be used, depending upon the cell type used.

X1.6.9.14 Remove media containing the toxin and dispose as recommended.

X1.6.9.15 Fix the cells for 30 min in 0.2 mL of PBS containing 10 % (wt/vol) formalin. Remove the formalin and dispose in accordance with your institution' chemical waste plan. Do not let the plate dry out before the next step.

X1.6.9.16 Stain the wells for approximately 1.5 h by adding 0.2 mL ethanol containing 0.13 % (wt/vol) crystal violet.

X1.6.9.17 Remove the residual stain by washing extensively with tap water. Avoid touching the walls of the plate with the pipette. Air-dry the plate.

X1.6.9.18 Solubilize the dye by adding 0.2 mL of 70 % (vol/vol) methanol in DI water.

X1.6.9.19 Shake the plate on an absorbance microtiter plate reader for 5 min and then measure the absorbance at 600 nm using a microplate absorbance instrument.

X1.6.9.20 *Interpretation of Results*—Record absorbance values from the microplate reader in an appropriate software program. Plot absorbance values (*y*-axis) versus ricin concentration (*x*-axis) The ID50 concentration for the ricin is determined where the curve intersects 50 % of the value between 0 cytoxicity (wells for control cells without toxin) and 100 % cytotoxicity. The values for 100 % cytoxicity should be those wells with a known toxin such as cyloheximide, but in practice the values from wells without added cells will be essentially the same low value. Low values (clear wells) are intoxicated cells, and high values (blue wells) indicate that the toxin has been neutralized or lack of toxin. Buffer control wells should be clear, and negative control wells with cells but no toxin should be blue.

X1.6.10 *Alternative Methods to Measure Cell Cytotoxicity or Inhibition of Protein Synthesis:*

X1.6.10.1 The incorporation of radioactive amino acids (eg. Leucine) into proteins can be used to measure protein synthesis. Ricin will inhibit the incorporation of the radioactive amino acid in proteins compared to cells without ricin. Dilutions of ricin prepared in media are added to cells and incubated for a suitable time (typically 18 h at 37°C with 5 % $CO_2$). Media is removed and media containing the radioactive amino acid to the wells and the plates incubated for an additional period of time (typically 4 h). Proteins are isolated from the cells and the extent of amino acid(s) incorporation is measured by the amount of radioactivity.

X1.6.10.2 *Assays Measuring Cell Viability and Toxicity*—The enzymatic substrate yellow tetrazolium MTT assay (ATCC, Manassas, VA, no. 30-1010K), the MultiTox-Fluor™ (MTox) multiplex cytotoxicity assay (Promega, Madison, WI), and the CellTiter-Glo® (CTG) luminescent cell viability assay (Promega) used to measure cytotoxicity of Vero cells in response to ricin have been compared (19).

### X1.7 Cell Line Preservation

X1.7.1 Label cryovials with cell type, catalog #, # cells/mL, passage #, date, and operator's initials.

X1.7.2 Freeze the cells in medium containing complete growth medium supplemented with 5 % (v/v) DMSO. Store frozen cells in liquid nitrogen vapor phase.

NOTE X1.1—DMSO is a powerful solvent, at all times care should be taken to avoid DMSO coming into contact with the skin.

X1.7.3 Alternatively, spin down the cells at 100X g for 10 min and remove the growth media. Resuspend the cell pellet in freezing media (90 % FBS and 10 % DMSO) to obtain a concentration of 1 by $10^6$ cells/mL. Add 1 mL aliquots of cells into cryovials and place vials in a storage unit that allows freezing at 1°C per minute at -80°C. After 24 h at -80°C, move cells to a freezer kept at -135°C or store cells in liquid nitrogen vapor phase.

X1.7.4 References for cell freezing.[18,19]

### X1.8 Report

X1.8.1 The identity of the cell used should be reported along with the composition of the media and conditions used for cell growth.

X1.8.2 The method used for cytotoxicity and the length of time the cells were contacted with ricin should be included.

[18] http://ncgc.nih.gov/guidance/section7.html#cryopreservation-cells
[19] http://www.hpacultures.org.uk/technical/ccp/procedureforfreezingcells

## X2. MEASUREMENT OF RICIN ENZYMATIC ACTIVITY USING SYNTHETIC SUBSTRATES

X2.1 The ricin A chain has N-glycosidase enzymatic activity that removes a specific adenine base in the 28S ribosomal RNA (rRNA) chain of the eukaryotic ribosomes, resulting in the inactivation of the ribosome and stopping protein synthesis (21, 22).

X2.2 Although the physiological target is rRNA in the ribosome, the N-glycosidase activity of ricin will catalyze the removal of an adenine base in synthetic RNA or DNA substrates that are present in a hairpin loop structure with the sequence GAGA. The first adenine in the GAGA loop is removed leaving an abasic site in the oligonucleotide. The choice of either ribose or deoxyribose nucleotides in the oligonucleotide used to form the stem loop structure of the substrate used can have a major effect on the kinetic parameters measured (23). For example, a deoxyadenine base in the second position of the RNA loop substrates (GdAGA) resulted in enhanced enzymatic rates (23, 24).

X2.3 To facilitate the comparison of results obtained with synthetic substrates to results obtained with other measurements, kinetic parameters of the assay should be measured. Consultation with general references on enzymatic measurements should be done to ensure reliable measurements of the kinetic parameters.

X2.3.1 The International Union of Biochemistry and Molecular Biology publishes recommendations for the enzyme nomenclature and descriptions of enzymatic activity parameters (23, 25).

X2.3.2 The catalytic constant ($K_{cat}$), the apparent first order rate constant of the reaction, represents the maximum number of reactions per second. The $K_{cat}$ should be measured at high concentrations of substrate to ensure that the substrate is saturated.

X2.3.3 Michaelis-Menten kinetics are used to model the behavior of enzymes such as ricin with a substrate and is a useful parameter to describe how well the A-chain binds the substrate. The $K_m$ is the substrate concentration at which the enzyme achieves one-half the maximum velocity ($V_{max}$). The kinetic parameters of an enzyme are dependent upon the substrate as well as the reaction conditions, such as temperature, pH, and potentially buffer components. For a more complete description of enzyme kinetics, see general references on enzymology (25, 26).

X2.3.4 The kinetic parameters using ricin's physiological substrate (the ribosome) has been measured. Ribosomes from rabbit reticulocytes gave $K_m$ values in the range of 100 nM and $K_{cat}$ values in the range of 1000 $min^{-1}$ (27, 28).

### X2.4 Measurement of Released Adenine

X2.4.1 Released adenine from the reaction can be measured using a colorimetric assay that reacts with phosphate (29), by measuring adenine directly using HPLC method ( 11) or after fluorescent derivatization (30).

X2.4.2 An assay has been developed based on the release of radioactive adenine from $^3$H-labeled plasmid DNA (31).

X2.4.3 Mass-spectroscopy has been used to detect adenine released from a synthetic RNA substrate (32).

### X2.5 Detection of the Depurinated Substrate

X2.5.1 The depurination of a synthetic RNA substrate can be measured by hybridization to a DNA molecular beacon (33). The extent of binding is determined by fluorescence measurements.

X2.5.2 A oligonucleotide probe containing a fluorescent nucleotide is used to measure the activity of RIP toxins using a synthetic substrate (34). The fluorescence of the probe molecule increases when hybridized to the abasic site in the substrate.

X2.5.3 An assay for RIP toxins utilizes the fact that reverse transcriptase will substitute an adenine into the opposite strand when the enzyme encounters an abasic site in RNA. The substitution was detected using quantitative polymerase chain reaction assay (35).

X2.5.4 A sensitive assay for ricin biological activity has been developed using electrochemiluminescence (ECL). This assay has also been applied to a limited number of food samples and two ECL platforms, one tube-based and the other employing a 96-well format (36-38). When ricin depurinates synthetic RNA substrates, the substrate is chemically cleaved and hybridized to a ruthenylated DNA oligonucleotide. ECL detection is then done on the complex that is immobilized on the plates or captured on the magnetic beads. A useful feature of the assay is the ability to detect and distinguish between different ribosome inactivating proteins (for example, abrin and ricin) based on the relative activity with synthetic oligonucleotide substrates of differing sequences (37).

X2.5.5 Matrix-assisted laser desorption/time of flight (MALDI-TOF) is used to measure the depurination by ricin of a DNA oligonucleotide substrate (39)

### X2.6 Reporting

X2.6.1 The following parameters of the ricin enzyme reaction conditions should be reported: (*1*) temperature, (*2*) pH, and (*3*) composition of the buffer.

X2.6.2 The following parameters of the synthetic substrate should be reported: (*1*) the structure, (*2*) the sequence, (*3*) concentration, and (*4*) molecular weight.

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